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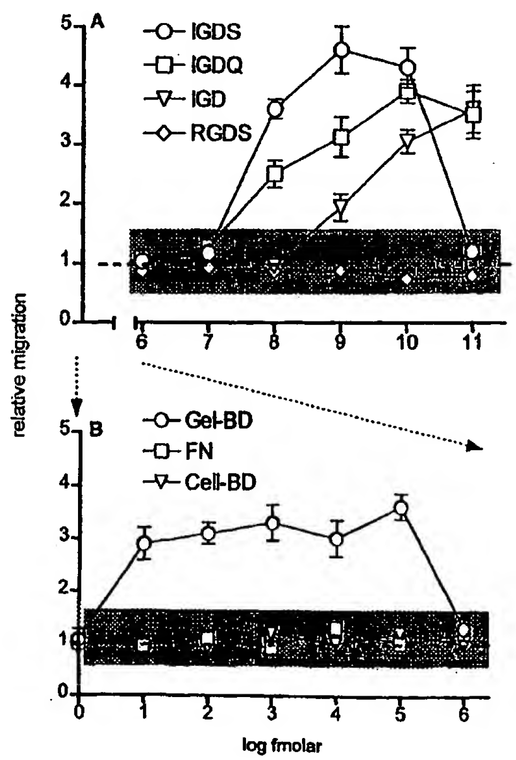
(54) Title: PEPTIDES CONTAINING THE MOTIF IGD AND THEIR USE AS CELL MIGRATION MODULATORS

(57) Abstract

A compound with a relative molecular mass of less than 15000 comprising the peptide Ile–Gly–Asp (IGD) or a peptide or non–peptide mimic thereof. The compounds may be used to modulate cell migration and are useful in angiogenesis.



PEPTIDES CONTAINING THE MOTIF IGD AND THEIR USE AS CELL MIGRATION MODULATORS

The present invention relates to peptides and related compounds and in particular to peptides and related compounds which affect cell migration.

Fibronectin is a widely distributed glycoprotein present at high concentrations in most extracellular matrices, in plasma (300 μg/ml), and in other body fluids. Fibronectin is a prominent adhesive protein and mediates various aspects of cellular interactions with extracellular matrices including migration. Its principal functions appear to be in cellular migration during development and wound healing, regulation of cell growth and differentiation, and haemostasis/thrombosis.

Fibronectin is a dimer of two non-identical subunits covalently linked near their COOH-termini by a pair of disulphide bonds. The difference between the subunits is determined by alternative splicing of the IIICS (or V) region. In the insoluble, matrix form of fibronectin, the dimer associates into disulphide-bonded oligomers and fibrils, while soluble, body fluid fibronectin is predominantly dimeric. Three regions of fibronectin are subject to alternative splicing and in general the matrix form of the molecule has a higher content of these segments than the soluble form. The human IIICS region has five potential variations, while the rat, bovine and chicken sequences have three, three and two, respectively. Each subunit is composed of a series of structurally independent domains linked by flexible polypeptide segments. At the primary sequence level, the origin of the majority of the fibronectin molecule can be accounted for by endoduplication of three types of polypeptide repeat. Different fibronectin domains are specialized for binding extracellular matrix macromolecules or bacterial or eukaryotic membrane receptors. The central cell-binding domain is recognised by

most adherent cells via the integrin receptors $\alpha3\beta1$, $\alpha5\beta1$, $\alpha V\beta1$, $\alpha IIb\beta3$, $\alpha V\beta3$, $\alpha V\beta5$ and $\alpha V\beta6$. The IIICS/HepII cell-binding domain is recognised by lymphoid cells, neural crest derivatives and myoblasts via the integrins $\alpha4\beta1$ and $\alpha4\beta7$. Several peptide active sites have been identified in these domains.

Plasma fibronectin can be purified by a combination of gelatin and heparin affinity chromatography. Cell-associated fibronectin can be extracted from culture monolayers with 1 M urea. Further details on fibronectin are in *The Extracellular Matrix Facts Book*, Ayad *et al* (eds), Academic Press, Harcourt Brace & Company, London.

Limited proteolytic digestion of fibronectin results in the release of a number of its functional domains which are characterised by their specific adhesion to other matrix macromolecules or integrin receptors on the cell surface (ie the cell-binding domain)[1]. The transmembrane assay has commonly been used to study the effects of fibronectin and its purified functional domains on cell migration *in vitro*. Essentially, this assay involves assessing cell movement through a polycarbonate membrane coated with an adhesive protein (usually gelatin) separating upper and lower medium compartments containing the putative effector molecule. Previous studies have revealed that nano- to micromolar concentrations of fibronectin and its purified cell-binding domain stimulate the migration of a wide range of cell types in the transmembrane assay[2,3]. Related studies implicated the RGD amino acid motif (located in the tenth type III repeat module) in mediating these effects of both native fibronectin and its cell-binding domain[3]. Significantly, small RGD-containing synthetic peptides did not stimulate cell migration; indeed, these peptides inhibited the adhesive and migration stimulating activity of larger protein domains containing the RGD motif by competition for receptor ligation[4]. In

contrast to the activity of the cell binding domain, the gelatin-binding domain of fibronectin has consistently been reported to be devoid of migration stimulating activity in the transmembrane assay[2,5].

Schor *et al* (1994) *Progress in Growth Factor Research* 5, 223-248 is a review of cytokine control of cell motility and its modulation and mediation by the extracellular matrix.

Schor *et al* (1993) In: *Cell Behaviour: Adhesion and Motility* (ed. G. Evans, C. Wigley and R. Warn) *Society of Experimental Biology Symposium No. 47*, pages 235-251 relates to migration stimulating factor (MSF).

Grey *et al* (1989) *Proc. Natl. Acad. Sci. USA* **86**, 2438-2442 relates to the purification of the MSF produced by fetal and breast cancer patient fibroblasts.

US 5,300,630 relates to oncodevelopmentally regulated antigens related to fibronectin.

US 5,510,328 relates to methods of reducing or inhibiting wound contraction using certain peptides.

US 5,354,736 relates to compounds that have enhanced cell binding with respect to collagen.

US 4,976,734 relates to a method of stimulating chemotaxis towards a prosthetic device.

US 4,980,279 relates to portions of fibronectin.

US 5,049,658 relates to a polypeptide having the cell-spreading activity of human fibronectin.

US 5,124,155 relates to wound healing dressings which are prepared by flocculating fibronectin.

US 5,453,489 relates to polypeptides which encompass fibronectin-fibronectin binding sites and which are capable of inhibiting fibronectin matrix assembly.

US 5,192,746 relates to compounds having the property of modulating cell adhesion.

US 5,491,130 relates to peptides derived from human endothelial cell thrombosponding which bind to the gelatin-binding domain of fibronectin.

We have previously developed an alternative migration assay involving the assessment of cell movement into gels of native type I collagen fibres[6]. Using this assay, we have recently reported that femtomolar concentrations of the gelatin-binding domain stimulated the migration of human dermal fibroblasts, whilst native fibronectin and its cell-binding domain were inactive[7].

We observed that the gelatin-binding domain did indeed stimulate cell migration in the transmembrane assay when filters were coated with native collagen, but not with gelatin (as used in the majority of previous studies). One of the objectives of the present study has been to determine whether a candidate amino acid sequence within the gelatin-binding domain of fibronectin is responsible for its substratum-dependent stimulation of cell migration and, if so, the manner in which it may be functionally related

to the RGD motif. Surprisingly we have found that peptides and other molecules containing the IGD amino acid sequence motif stimulate fibroblast migration into native but not denatured collagen substrate.

A first aspect of the invention provides a compound with a relative molecular mass of less than 15 000 comprising the peptide Ile-Gly-Asp (IGD) or a peptide or non-peptide mimic thereof.

Preferably, the compound has a relative molecular mass of less than 12000, more preferably less than 10000.

We have found that, surprisingly, the peptide IGD alone or when present as a moiety in a larger molecule is able to modulate cell migration. Thus, the preferred compounds of the invention are those which are able to modulate cell migration under appropriate conditions such as those conditions described in the Examples.

By peptide mimic of IGD we include that the Ile is replaced by another hydrophobic amino acid such as Val, Leu, Phe, Trp or Tyr, most preferably Val or Leu. We also include that the Asp is replaced by Glu. Less preferably the Gly residue is replaced by Ala. The peptide mimics are preferably those that exhibit substantially the same cell migration moduluating activity of a peptide comprising the peptide sequence IGD and, more preferably substantially the same cell migration modulating activity of any one of the peptides IGD or IGDS or IGDQ. Suitably, the peptide mimic comprises a moiety which has substantially the same charge distribution and/or spatial configuration as any one of the peptides IGD, IGDS or IGDQ.

By non-peptide mimics of IGD we include a moiety which has the same

charge distribution and/or spatial configuration as any one of the peptides IGD, IGDS or IGDQ, and we include a moiety which has substantially the same cell migration modulating activity of a peptide comprising the IGD sequence.

Non-peptide surrogates of the RGD sequence have been developed or are described by, for example, Greenspoon *et al* (1993) *Biochemistry* **32**, 1001-1008 and Humphries *et al* (1994) *Exp. Opin. Ther. Patents* **4**, 227-235, both of which are incorporated herein by reference, and suitable non-peptide mimics based on the IGD motif can be made using similar principles as for the non-peptide mimics of RGD.

Compounds which exhibit substantially the same cell migration modulating activity of a peptide comprising the peptide Ile-Gly-Asp (IGD) can be selected using a suitable screening system. The preferred screening system uses a migration assay similar to that described in Example 1 in which cell migration is assessed on a native type I collagen substratum (for example, collagen gel or transmembrane assay using collagen-coated membranes). Mimics are compounds which exhibit substantially the same effect as the peptides IGD, IGDS or IGDQ and which may act in an additive fashion with them. Inhibitors would abrogate the bioactivity of the peptides IGD, IGDS or IGDQ. The peptide IGD and the gel binding domain (GBD) act in an additive fashion, whereas the RGD peptide is an inhibitor of the IGD peptide.

Although the peptide sequence IGD, when present in a compound of the invention, is sufficient to modulate cell migration it is preferred if the peptide sequence Ile-Gly-Asp-Ser (IGDS) or Ile-Gly-Asp-Gln (IGDQ) is present in a compound of the invention.

We have found, in relation to the peptides IGDS, IGDQ and IGD that IGDS is more potent than IGDQ which is more potent than IGD in stimulating fibroblast migration and so it is preferred if the compound comprises the peptide IGDS.

It is preferred that the amino acids within the peptide sequence IGD or IGDS or IGDQ, when present in the compounds of the invention, are all in the L configuration as is the case for natural amino acids. It will, nevertheless, be appreciated that when the compound of the invention is a peptide comprising IGD or IGDS or IGDQ then the other amino acids within the peptide (ie those other than the ones in the IGD or IGDS or IGDQ moiety) may be in the L- or D- configurations. In as much as peptides containing D-amino acids may be more resistant to proteolysis compounds containing D-amino acids (other than in the IGD or IGDS or IGDQ moiety) may be preferred.

The invention covers all compounds with a relative molecular mass of less than 15 000 comprising the peptide IGD or a peptide or non-peptide mimic thereof; however, it is particularly preferred if the compound is a peptide comprising the peptide moiety IGD. In other words, preferred compounds are peptides larger than 3 amino acids which contain the peptide moiety IGD. Such peptides include the peptides IGDS and IGDQ and also include peptides with additional amino acids N terminal and/or C terminal to these motifs.

The compounds of the invention also include peptides wherein the IGD or IGDS or IGDQ moiety is masked by, for example, blocking groups being present on free -NH_2 or -COOH groups. Preferably, such blocking groups are ones which may be removed readily *in vivo*, for example by hydrolysis; however, in some circumstances it may be desirable if the

blocking groups are substantially resistant to hydrolysis.

The peptide IGD is also a compound of the invention.

It is preferred if the compounds have a relative molecular mass of less than 8000, preferably less than 6000, more preferably less than 5000, and preferably less than 2000. When the compound of the invention is a peptide it is preferred if the peptide has between 4 and 80 amino acid residues, more preferably between 4 and 50, still more preferably between 4 and 30 and preferably between 4 and 20 amino acid residues.

The compound of the invention may have a linear configuration or it may be branched or circular. When the compound of the invention is a peptide it may be linear, branched or circular.

It will be appreciated that the compound of the invention may comprise more than one IGD peptide moiety or a peptide or non-peptide mimic thereof. In certain circumstances it is advantageous for the compound of the invention to comprise between two and 50 such peptide moieties or a peptide or non-peptide mimics thereof, more preferably between 2 and 20 and most preferably between 5 and 15 such peptide moieties or peptide or non-peptide mimics thereof.

Thus, the peptide of the invention may consist of multiple repeats of IGD or IGDS or IGDQ or a peptide sequence containing conservative substitutions for I, G or D (such as IGES), and it may consist of any of these, including multiple repeats, in a cyclic form.

When the compound of the invention is a peptide it is possible for it to contain tandem repeats of the IGD-containing moiety (such as IGD itself

or IGDS or IGDQ or combinations thereof).

Although not essential, it is preferred if the peptide of the invention comprises the IGD motif and flanking regions from the fibronectin molecule. For example, the flanking regions may be 1, 2, 3, 4, 5 or more amino acid residues on one or both sides of the occurrence of IGD in the fibronectin molecule. The amino acid sequence of the human fibronectin molecule is given in Figure 5. Peptides containing flanking regions of fibronectin may have greater bioactivity than shorter tri- and tretra-peptides. These flanking regions are preferably derived from the seventh (for IGDQ) and ninth (for IGDS) type I repeat modules for fibronectin. It is also preferred if the compounds of the invention are the intact fibronectin type I repeat modules (I_7 and I_9) since these may exhibit higher bioactivity than shorter synthetic peptides. Each of these type I modules contains approximately 45 amino acids and, preferably up to three of these modules are used in tandem array.

When the compound of the invention is a peptide it may be synthesised using well known methods in the art. For example, peptides may be synthesised by the Fmoc-polyamide mode of solid-phase peptide synthesis as disclosed by Lu *et al* (1981) *J. Org. Chem.* **46**, 3433 and references therein. Temporary N-amino group protection is afforded by the 9-fluorenylmethyloxycarbonyl (Fmoc) group. Repetitive cleavage of this highly base-labile protecting group is effected using 20% piperidine in N,N-dimethylformamide. Side-chain functionalities may be protected as their butyl ethers (in the case of serine threonine and tyrosine), butyl esters (in the case of glutamic acid and aspartic acid), butyloxycarbonyl derivative (in the case of lysine and histidine), trityl derivative (in the case of cysteine) and 4-methoxy-2,3,6-trimethylbenzenesulphonyl derivative (in the case of arginine). Where glutamine or asparagine are C-terminal

residues, use is made of the 4,4'-dimethoxybenzhydryl group for protection of the side chain amido functionalities. The solid-phase support is based on a polydimethyl-acrylamide polymer constituted from the three monomers dimethylacrylamide (backbone-monomer), bisacryloylethylene diamine (cross linker) and acryloylsarcosine methyl ester (functionalising agent). The peptide-to-resin cleavable linked agent used is the acid-labile 4-hydroxymethyl-phenoxyacetic acid derivative. All amino acid derivatives are added as their preformed symmetrical anhydride derivatives with the exception of asparagine and glutamine, which are added using a reversedN,N-dicyclohexyl-carbodiimide/1-hydroxybenzotriazolemediated coupling procedure. All coupling and deprotection reactions are monitored using ninhydrin, trinitrobenzene sulphonic acid or isotin test procedures. Upon completion of synthesis, peptides are cleaved from the resin support with concomitant removal of side-chain protecting groups by treatment with 95% trifluoroacetic acid containing a 50% scavenger mix. Scavengers commonly used are ethanedithiol, phenol, anisole and water, the exact choice depending on the constituent amino acids of the peptide being synthesised. Trifluoroacetic acid is removed by evaporation *in vacuo*, with subsequent trituration with diethyl ether affording the crude peptide. Any scavengers present are removed by a simple extraction procedure which on lyophilisation of the aqueous phase affords the crude peptide free of scavengers. Reagents for peptide synthesis are generally available from Calbiochem-Novabiochem (UK) Ltd, Nottingham NG7 2QJ, UK. Purification may be effected by any one, or a combination of, techniques such as size exclusion chromatography, ion-exchange chromatography and (principally) reverse-phase high performance liquid chromatography. Analysis of peptides may be carried out using thin layer chromatography, reverse-phase high performance liquid chromatography, amino-acid analysis after acid hydrolysis and by fast atom bombardment (FAB) mass spectrometric analysis.

Alternatively, when the peptide of the invention is of a suitable size, such as greater than about 50 residues in length, it may be desirable to produce the peptide by recombinant DNA technology.

The peptides of the invention may be encoded by a suitable polynucleotide which may be obtained or synthesised by methods well known in the art.

The DNA is then expressed in a suitable host to produce a peptide comprising the compound of the invention. Thus, the DNA encoding the peptide constituting the compound of the invention may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate host cell for the expression and production of the peptide of the invention. Such techniques include those disclosed in US Patent Nos. 4,440,859 issued 3 April 1984 to Rutter *et al*, 4,530,901 issued 23 July 1985 to Weissman, 4,582,800 issued 15 April 1986 to Crowl, 4,677,063 issued 30 June 1987 to Mark *et al*, 4,678,751 issued 7 July 1987 to Goeddel, 4,704,362 issued 3 November 1987 to Itakura *et al*, 4,710,463 issued 1 December 1987 to Murray, 4,757,006 issued 12 July 1988 to Toole, Jr. *et al*, 4,766,075 issued 23 August 1988 to Goeddel *et al* and 4,810,648 issued 7 March 1989 to Stalker, all of which are incorporated herein by reference.

The DNA encoding the peptide constituting the compound of the invention may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether episomal maintenance or integration is desired.

Generally, the DNA is inserted into an expression vector, such as a

plasmid, in proper orientation and correct reading frame for expression. If necessary, the DNA may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the desired host, although such controls are generally available in the expression vector. The vector is then introduced into the host through standard techniques. Generally, not all of the hosts will be transformed by the vector. Therefore, it will be necessary to select for transformed host cells. One selection technique involves incorporating into the expression vector a DNA sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such as antibiotic resistance. Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression of the peptide, which can then be recovered.

Many expression systems are known, including bacteria (for example *E. coli* and *Bacillus subtilis*), yeasts (for example *Saccharomyces cerevisiae*), filamentous fungi (for example *Aspergillus*), plant cells, animal cells and insect cells.

The vectors include a prokaryotic replicon, such as the ColE1 *ori*, for propagation in a prokaryote, even if the vector is to be used for expression in other, non-prokaryotic, cell types. The vectors can also include an appropriate promoter such as a prokaryotic promoter capable of directing the expression (transcription and translation) of the genes in a bacterial host cell, such as *E. coli*, transformed therewith.

A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with exemplary bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention.

Typical prokaryotic vector plasmids are pUC18, pUC19, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA, USA) and p*Trc*99A and pKK223-3 available from Pharmacia, Piscataway, NJ, USA.

A typical mammalian cell vector plasmid is pSVL available from Pharmacia, Piscataway, NJ, USA. This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells.

An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia. This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *HIS3, TRP1, LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps)

A variety of methods have been developed to operably link DNA to vectors via complementary cohesive termini. For instance, complementary homopolymer tracts can be added to the DNA segment to

be inserted to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion as described earlier, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3'-single-stranded termini with their 3'-5'-exonucleolytic activities, and fill in recessed 3'-ends with their polymerizing activities.

The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies Inc, New Haven, CN, USA.

A desirable way to modify the DNA encoding the polypeptide of the invention is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* **239**, 487-491.

In this method the DNA to be enzymatically amplified is flanked by two specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

The present invention also relates to a host cell transformed with a polynucleotide vector construct of the present invention. The host cell can be either prokaryotic or eukaryotic. Bacterial cells are preferred prokaryotic host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains DH5 available from Bethesda Research Laboratories Inc., Bethesda, MD, USA, and RR1 available from the American Type Culture Collection (ATCC) of Rockville, MD, USA (No ATCC 31343). Preferred eukaryotic host cells include yeast and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line. Yeast host cells include YPH499, YPH500 and YPH501 which are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Preferred mammalian host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658, and monkey kidney-derived COS-1 cells available from the ATCC as CRL 1650.

Transformation of appropriate cell hosts with a DNA construct of the present invention is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of prokaryotic host cells, see, for example, Cohen *et al* (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2110 and Sambrook *et al* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. Transformation of yeast cells is described in Sherman *et al*

(1986) *Methods In Yeast Genetics, A Laboratory Manual*, Cold Spring Harbor, NY. The method of Beggs (1978) *Nature* **275**, 104-109 is also useful. With regard to vertebrate cells, reagents useful in transfecting such cells, for example calcium phosphate and DEAE-dextran or liposome formulations, are available from Stratagene Cloning Systems, or Life Technologies Inc., Gaithersburg, MD 20877, USA.

Electroporation is also useful for transforming cells and is well known in the art for transforming yeast cell, bacterial cells and vertebrate cells.

For example, many bacterial species may be transformed by the methods described in Luchansky *et al* (1988) *Mol. Microbiol.* 2, 637-646 incorporated herein by reference. The greatest number of transformants is consistently recovered following electroporation of the DNA-cell mixture suspended in 2.5X PEB using 6250V per cm at 25μFD.

Methods for transformation of yeast by electroporation are disclosed in Becker & Guarente (1990) *Methods Enzymol.* **194**, 182.

Successfully transformed cells, ie cells that contain a DNA construct of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an expression construct of the present invention can be grown to produce the polypeptide of the invention. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* **98**, 503 or Berent *et al* (1985) *Biotech.* 3, 208. Alternatively, the presence of the protein in the supernatant can be detected using antibodies as described below.

In addition to directly assaying for the presence of recombinant DNA,

successful transformation can be confirmed by well known immunological methods when the recombinant DNA is capable of directing the expression of the protein. For example, cells successfully transformed with an expression vector produce proteins displaying appropriate antigenicity. Samples of cells suspected of being transformed are harvested and assayed for the protein using suitable antibodies.

Thus, in addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium.

Thus, a second aspect of the invention provides a polynucleotide encoding a peptide of the invention.

A third aspect of the invention provides a vector comprising a polynucleotide of the invention and a fourth aspect of the invention provides a host cell comprising a polynucleotide or vector of the invention.

The compounds of the invention are useful in modulating cell migration and therefore are useful in medicine.

Thus, a fifth aspect of the invention provides a compound according to the first aspect of the invention for use in medicine.

A sixth aspect of the invention provides a pharmaceutical composition comprising a compound according to the first aspect of the invention and a pharmaceutically acceptable carrier.

The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient (compound of the invention) with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations in accordance with the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets, each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (eg povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (eg sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethylcellulose in varying proportions to provide desired release profile.

Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavoured basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouth-washes comprising the active ingredient in a suitable liquid carrier.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage formulations are those containing a daily dose or unit, daily sub-dose or an appropriate fraction thereof, of an active ingredient.

It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example those suitable for oral administration may include flavouring agents.

It is particularly preferred if the formulation is for topical administration,

for example to the site of a wound.

It will be appreciated that some of the compounds of the invention will be in the form of salts.

Salts which may be conveniently used in therapy include physiologically acceptable base salts, for example, derived from an appropriate base, such as an alkali metal (eg sodium), alkaline earth metal (eg magnesium) salts, ammonium and NX_4^+ (wherein X is C_{1-4} alkyl) salts. Physiologically acceptable acid salts include hydrochloride, sulphate, mesylate, besylate, phosphate and glutamate.

Salts according to the invention may be prepared in conventional manner, for example by reaction of the parent compound with an appropriate base to form the corresponding base salt, or with an appropriate acid to form the corresponding acid salt.

The aforementioned compounds of the invention or a formulation thereof may be administered by any conventional method including oral and parenteral (eg subcutaneous or intramuscular) injection. The treatment may consist of a single dose or a plurality of doses over a period of time.

Whilst it is possible for a compound of the invention to be administered alone, it is preferable to present it as a pharmaceutical formulation, together with one or more acceptable carriers. The carrier(s) must be "acceptable" in the sense of being compatible with the compound of the invention and not deleterious to the recipients thereof. Typically, the carriers will be water or saline which will be sterile and pyrogen free.

A further aspect of the invention provides a method of modulating cell

migration the method comprising administering an effective amount of a compound according to the first aspect of the invention to the site where modulation of cell migration is required.

Preferred compounds are those preferred in the first aspect of the invention.

Cell migration may be modulated according to the method of this aspect of the invention *in vitro*, for example in cell culture systems, or it may be modulated *in vivo*.

Impaired cell migration is commonly a feature of clinical conditions in which wound healing is not optimal; the stimulation of cell migration under these conditions may prove beneficial. Conversely, elevated or inappropriate cell migration is a feature of several pathological conditions, including tumour invasion, pathological angiogenesis, inflammation and fibrosis. Inhibitors of IGD bioactivity may prove useful in the treatment of these conditions. Inhibitors of IGD bioactivity may be screened for using method apparent to the skilled person based on the information contained herein.

The modulation of cell migration is desirable in, for example, wound healing, guided periodontal tissue regeneration, inhibition of tumour invasion and metastasis, and the compounds of the invention are also useful because of their effects on angiogenesis (new blood vessel formation). The compounds of the invention may also be useful in relation to inflammation or connective tissue function.

Thus, it is preferred that the site where modulation of cell migration is in an animal body, for example a mammalian, especially human, body. It

is also preferred if the cell whose migration is modulated is a fibroblast cell. We have shown that vascular cells are responsive to certain compounds of the invention (for example, IGDS), and that IGDS stimulates angiogenesis in the chick yolk sac assay. Thus, the compounds of the invention are believed to be clinically useful in stimulating angiogenesis in conditions such as impaired wound healing.

It will be seen, therefore, that the invention includes a method of treating an animal, for example a mammal, especially human, in need of modulation of cell migration the method comprising administering to the animal an effective amount of a compound according to the first aspect of the invention.

A further aspect of the invention provides the use of a compound of the invention for modulating cell migration, especially in wound healing or periodontal tissue regeneration or inhibition of tumour invasion and metastasis or in modulating angiogenesis.

A still further aspect of the invention therefore provides use of a compound of the invention in the manufacture of a medicament for modulating cell migration in an animal body.

The invention will now be described in more detail with reference to the following Figures and Examples in which:

Figure 1 shows the effects of synthetic peptides and fibronectin domains on cell migration in the native collagen gel assay. Assays were performed as described in Materials and Methods in Example 1. Results are normalised by expressing them as "relative stimulation" of migration, this being calculated by dividing the percentage of cells in the gel matrix for

each experimental point by the control value obtained in that particular experiment. Data are presented as the mean ± SD of five experiments. For clarity, the shaded area indicates the spread of standard deviations for data points not plotted with error bars. Upper panel: results obtained with the indicated synthetic peptides. Lower panel: results obtained with native fibronectin (FN), and its cell-binding (Cell-BD) and gelatin-binding (Gel-BD) domains.

Figure 2 shows the effects of synthetic peptides and fibronectin domains on cell migration in the transmembrane assay. Assays were performed as described in Materials and Methods in Example 1. Results from five experiments are presented as mean ± SD. Shaded area indicates the spread of standard deviations for data points not plotted with error bars. Panel A: results obtained with IGDS and the gelatin-binding domain (Gel-BD) on membranes coated with native collagen and gelatin. Panel B: results obtained with RGDS, native fibronectin (FN), and its cell-binding domain (Cell-BD).

Figure 3 shows the effect of different peptides on the migration of stimulating activity of the gelatin-binding domain of fibronectin and IGDS. The collagen gel assay was performed in the presence of various combinations of the indicated peptides and the percentage of cells present within the gel matrix measured after a four day incubation period. Data are expressed as mean ± SD obtained in three experiments. Panel A: combinations of the gelatin-binding domain (Gel-BD) and IGDS; Panel B: combinations of IGDS and RGDS; Panel C: combinations of IGDS and the cell-binding domain (Cell-BD).

Figure 4 shows the effect of pre-incubation of cells with synthetic peptides on their subsequent migration in the native collagen gel assay. Confluent

cells on plastic tissue culture dishes were washed 3x with serum-free MEM (SF-MEM) and then incubated for various times with the indicated concentrations of IGDS in SF-MEM. They were then trypsinised and washed extensively by repeated (5x) cycles of centrifugation and resuspension in SF-MEM. The behaviour of these pre-incubated cells was assessed in the collagen gel assay in the absence of further IGDS (indicated as *IGDS not in assay*). These results were compared with the response of control cells to IGDS present during the four day duration of the migration assay (indicated as *IGDS in assay*). Data were obtained from three experiments and are expressed as mean ± SD.

Figure 5 shows the primary amino acid structure of human fibronectin.

Primary structure of Fibronectin

Ala A 100	Cys C 63	Asp D 126	Glu E 145
Phe F 54	Gly G 208	His H 51	Ile I 121
Lys K 78	Leu L 136	Met M 27	Asn N 101
Pro P 195	Gln Q 133	Arg R 126	Ser S 200
Thr T 268	Val V 200	Trp W 40	Tyr Y 105
Mol. Wt (calc.) = 273 715		Residues = 2476	

Structural sites

Signal peptide: 1-20

Propeptide: 21-31

Type I repeats: 52-96, 97-140, 141-185, 186-230, 231-272, 308-344, 470-517, 518-560, 561-608, 2297-2341, 2342-2385, 2386-2428

Type II repeats: 345-404, 405-469

Type III repeats: 609-700, 719-809, 810-905, 906-995, 996-1085, 1086 1172, 1173-1265, 1357-1447, 1448-1537, 1538-1631, 1632-1721, 1812-1903-, 1904-1992, 1993-2082, 2203-2273

Alternatively spliced domains: 1722-1811 (ED-A), 1266-1356 (ED-B), 2083-2202 (IIICS)

Potential N-linked glycosylation sites: 430, 528, 542, 877, 1007, 1244, 1291, 1904, 2199

O-Linked glycosylation site: 2155

Interchain disulphide bond residues: 2458, 2462

RGD cell adhesion site: 1615-1618

IDAPS cell adhesion site: 1994-1998

LDV cell adhesion site: 2102-2104

REDV cell adhesion site: 2182-2185

Heparin-binding sites: 2028-2046 (FN-C/H I), 2068-2082 (FN-C/H II)

Factor XIIIa transglutaminase cross-linking site: 34

Figure 6 shows the modulation of the effect of GBD and the IGDS tetrapeptide on fibroblast migration into 3D collagen matrices by cell density. Cells were plated at either confluent (conf) or subconfluent (subconf) densities. Shaded area indicates range of control values.

Figure 7 shows the angiogenic activity of IGDS synthetic peptide and gelatin-binding domain of fibronectin in the chick yolk sac membrane assay. Dried methylcellulose pellets containing the test samples and control pellets (lacking the test samples) were placed on the yolk sac membrane of 6 day chick embryos, as described in Materials and Methods in Example 1. The elicited reaction was checked after six hours

and assessed after 24 hr by observation of living embryos with a stereomicroscope. A. negative angiogenic response (in this case, elicited by control pellet); B. typical positive angiogenic response (in this case, elicited by pellet containing 1.0 ì g IGDS). After assessment at 24 hr, selected membranes were fixed in 2.5% EM grade glutaraldehyde in 0.2 M phosphate buffer, pH 7.4. These preparations were then stained with

1% toluidine blue and photographed using a Leica DM LB microscope. C. appearance of fixed and stained positive angiogenic response (in this case, elicited by pellet containing 1.0 μg of the gelatin-binding domain). Bar = 250 μm.

Example 1: Migration stimulating activity of the IGD amino acid motif

Summary

The gelatin-binding domain of fibronectin stimulates fibroblast migration into matrices of native type I collagen, but is devoid of such activity with cells adherent to a denatured collagen substratum. We now demonstrate the IGD motif, present at two sites within the gelatin-binding domain, displays the same substratum-dependent activity. Micromolar concentrations of IDG-containing synthetic peptides stimulated fibroblast migration into native (but not denatured) collagen substrata in the following activity order: IGDS>IGDQ>IGD. The related RGDS peptide did not affect cell migration on its own and inhibited the bioactivity of IGDS in a dose-dependent fashion. Cells pre-incubated with IGDS displayed a persistent stimulation of cell migration when assayed in the absence of further IGDS. This feature of IGDS bioactivity provided a means to study the early events of IGDS action (for example, receptor ligation and post-ligation signalling) separately from the late events resulting in the persistent stimulation of cell migration. Accordingly, experiments in which cells were incubated with IGDS and inhibitors in various temporal combinations indicated that (a) both early and late events of IGDS action were effectively inhibited by RGDS, as well as function-blocking antibodies to integrin subunits (β_1 and β_3) and heterodimer $\alpha_v\beta_3$, (b) neutralising antibodies to the "classic" fibronectin-binding $\alpha_5\beta_1$ integrin

were without effect, and (c) inhibition of tyrosine kinase activity blocked early events of IGDS action, inhibition of MAP kinase kinase blocked both early and late events, whilst inhibition of PKA only affected late events. *In vivo* studies further indicated that IGDS synthetic peptide elicited an angiogenic response in the chick yolk sac membrane; in contrast, RGDS and RGES peptides were inactive under the same experimental conditions. The expression of biological activity by IDG synthetic peptides (both *in vitro* and *in vivo*) stands in marked contrast to the inactivity of their well-studied RGDS counterparts and opens the possibility of developing a novel family of clinically relevant agents.

Materials and Methods

Chemicals. The synthetic peptides were prepared to greater than 99% purity in the Department of Biochemistry, University of Dundee. Fibronectin and its cell-binding and gelatin-binding domains were purified as previously described[7]. Monoclonal antibodies to the integrin subunits $\alpha2$ (cat. no. MCA743) and $\beta1$ (MCA1188) were supplied by Serotec (Oxford, UK); antibodies to $\beta3$ (MAB1957), $\alpha v\beta3$ (MAB1976) and $\alpha5\beta1$ (MAB 1969) were supplied by Chemicon (Harrow, UK); antibody to $\alpha5\beta1$ (M0604) was supplied by Dako (High Wycombe, UK). Genistein (cat. no. 34583-Q) and PD98059 (cat. no. 178278-Q) were purchased from Calbiochem, Nottingham. PKA inhibitor peptide (cat. no. P6062) was purchased from Sigma Chemical Co (Poole, Dorset, UK).

Cells. Experiments were performed with two lines of human skin fibroblasts (SK319 and FSF44, between passage 10-18) shown to be free of mycoplasmal contamination by staining with *Hoechst 33256*. Identical results were obtained with both lines and these cells are consequently not individually identified in the Figures. Stock cultures were maintained in

Eagle's Minimal Essential (MEM), as previously described[7].

Migration Assays. In the collagen gel assay, pre-formed 2 ml gels were overlaid with 1 ml of serum-free MEM (controls) or serum-free MEM containing the requisite concentration of effector molecule to give the desired final concentration. Trypsinised fibroblasts were suspended in serum-free MEM to give an inoculum containing 2×10^5 cells/ml and 1 ml of this was plated onto replicate control and test gels. After a 4 day incubation period at 37°C, the cells on the surface and within the 3D matrix of the gel were counted in 15 randomly selected fields by microscopic observation and these data used to calculate the percentage of total cells present within the gel matrix[6].

Polycarbonate membranes used in the transmembrane assay were immersed in an aqueous solution containing 10 μg/ml of either native type I collagen or heat-denatured type I collagen (gelatin) overnight at 37°C and then air-dried. Assays were performed as previously described[7].

Chick yolk sac membrane angiogenesis assay. The assay was performed essentially as described by Gush *et al* (1990) *J. Med. Engineer. Tech.* **14**, 205-209. Accordingly, four day old fertilised eggs were cracked in a tumbler, covered with a Petri dish and incubated at 37°C. Two days later dried methylcellulose pellets containing the test samples and control pellets (lacking the test samples) were placed on the yolk sac membrane. The elicited angiogenic reaction was assessed after 24 hr by observation of living embryos with a stereomicroscope. Selected membranes were fixed in 2.5% EM grade glutaraldehyde in 0.2 M phosphate buffer (pH 7.4), dissected, stained with 1% toluidine blue and mounted on glass slides for photomicroscopy.

Results and Discussion

Inspection of the amino acid sequence of the gelatin-binding domain revealed that it contains two IGD motifs located in the seventh and ninth type I repeat modules, respectively. This is of particular interest, as the IGD motif is a highly conserved feature of the type I module[8] and its location at the apex of the main type I loop is homologous to that of the RGD motif in the tenth type III repeat[9]. Relevant IGD-containing synthetic peptides were synthesised and their effect on the migration of human dermal fibroblasts examined in the collagen gel assay. Results summarised in Figure 1A indicate that IGDS (as present in the ninth type I module), IGDQ (as present in the seventh type I module) and IGD stimulated cell migration into native type I collagen gels in a dose-dependent fashion. Significant bioactivity was expressed by IGDS and IGDQ at a concentration of 0.1 μM, whilst comparable activity was first manifest by IGD at 10-100 μM. IGDS produced a bell-shaped dose-response; this was not obtained with either IGDQ or IGD within the concentration range examined. The structurally related RGDS tetrapeptide was devoid of migration stimulating activity in the native collagen gel assay. Comparative results obtained with purified, proteolytically-generated, fibronectin fragments (Figure 1B) confirmed our previous observations that the gelatin-binding domain exhibits significant migration stimulating activity, whilst native fibronectin and its purified cell-binding domain are inactive when tested within the same concentration range[7]. Comparison of data presented in Figures 1A and B further indicate that the micromolar concentration range of IGD-containing synthetic peptides required to induce a stimulation of cell migration is many orders of magnitude greater than the corresponding femtomolar concentration range of the larger gelatin-binding domain. These observations suggest the involvement of other amino acid motifs within the gelatin-binding domain

in facilitating recognition and/or binding of IGD to its putative cell surface receptor. In this context, Aota *et al*[10] reported that the PHSRN sequence in the ninth type III module of the cell binding domain is such a "synergistic" motif for RGD-dependent biological activity.

The effects of a native and denatured type I collagen substratum on the migration stimulating activity of IGD-containing synthetic peptides was assessed in the transmembrane assay. Data presented in Figure 2A indicate that IGDS stimulated cell migration through membranes coated with native collagen, but was devoid of activity on gelatin-coated membranes. Similar results were obtained with IGDQ and IGD (data not shown). These observations indicate that the bioactivity of IGD-containing synthetic peptides is (a) dependent upon cell attachment to a native collagen substratum, and (b) resembles that of the larger gelatin-binding domain in which it is contained in terms of this criterion. The mechanism responsible for the substratum-dependent nature of IGD activity remains to be determined. In this regard, it may be relevant that cellular adhesion to a native collagen substratum specifically affects a number of cellular processes of potential relevance to the modulation of cell migration (for example, phosphorylation of pp125FAK and activation of PKC-ζ)[11,12].

Data presented in Figure 2B confirm that native fibronectin and its purified cell binding domain stimulate cell migration through membranes coated with gelatin (as reported in previous studies) and that the RGDS synthetic peptide is inactive on both collagen- and gelatin-coated membranes.

The possible mechanistic relationship between IGDS and the gelatin-binding domain in which it is contained was further examined by co-incubating cells with suboptimal concentrations of each. The results

presented in Figure 3A indicate that these two peptides exerted an additive effect upon cell migration, consistent with the hypothesis that the IGD motif within the gelatin-binding domain is indeed responsible for its stimulation of cell migration. This additive effect was particularly apparent at 0.1 fM GBD and 0.01 μM (10^7 fM) IGDS, which were each inactive when present on their own, but active in combination.

Possible mechanistic interactions between IGDS and RGDS were studied in similar co-incubation experiments. Results presented in Figure 3B indicate that RGDS effectively inhibited the migration stimulating activity of IGDS. The cell-binding domain of fibronectin (which contains the RGDS motif) also inhibited IGDS activity (Fig 3C). As expected, the inhibition of IGDS activity was achieved at considerably lower (nanomolar) concentrations of the cell-binding domain compared to the μmolar concentrations required of RGDS. The RGES synthetic peptide had no effect on IGDS migration stimulating activity when tested at the same concentration range as RGDS (data not shown).

A number of biological activities of fibronectin are "cryptic" in the sense that they are displayed by fibronectin proteolytic fragments, but not by the intact molecule[5,13]. Fukai *et al*[14] have demonstrated that the expression of these cryptic activities require either the denaturation of native fibronectin and/or its limited proteolytic degradation into functional domains in order to become manifest. These authors suggest that relaxation of steric hindrance may be responsible for the unmasking of latent biological activity by these procedures. The inhibition of IGDS-induced cell migration by the RGDS amino acid motif may provide an additional mechanism for the apparent lack of IGDS activity in native fibronectin (Figure 1B).

We have previously reported that cells pre-incubated for 24 hr with the gelatin-binding domain of fibronectin displayed elevated migratory activity when subsequently plated on native collagen gels in its absence[7]. Data presented in Figure 4 indicate that the effect of IGDS on cell migration is similarly persistent, exhibiting a dependence upon both the time of pre-incubation and peptide concentration. This elevated migratory behaviour is still manifest by pre-incubated cells following 1-2 passages *in vitro* (data not shown).

The persistence of IGDS-bioactivity suggests that its mode of action involves a series of early events which are dependent upon the presence of IGDS (such as receptor ligation and post-ligation signalling) and later events which no longer require the presence of IGDS and ultimately result in stabilisation of a persistent migratory phenotype. The specific effects of potential inhibitory molecules on such early and late events were examined by using the following experimental protocol: (a) pre-incubating cells with IGDS and inhibitor and then assaying these treated cells in the absence of both IGDS and inhibitor (*note: this protocol provides data concerning the effects of inhibitor on the early events mediating IGDS activity*), (b) pre-incubating cells with neither IGDS nor inhibitor and assaying them in the presence of both (*the effects of inhibitor on both early and late events*), (c) pre-incubating cells with IGDS alone and then assaying them in the presence of inhibitor only (*the effects of inhibitor on late events*), and finally, (d) pre-incubating cells with inhibitor alone and then assaying them in the presence of IGDS *to provide control information regarding possibly persistent effects of inhibitor which would confound data interpretation.* Data presented in Table 1A are concerned with the effects of the synthetic RGDS and RGES. Our results indicate that RGDS inhibited both IGDS cell signalling and subsequent cell migration. RGES was inactive under all experimental conditions. Control data (protocol

"d") indicated that both peptides had no persistent effect on IGDS activity; all of the other inhibitors examined were similarly devoid of such potentially confounding activity (data not shown).

The inhibitory effects of RGDS on IGDS-induced cell migration may occur by competition for receptor ligation. In order to obtain data relevant to this possibility, we employed the above pre-incubation protocols to examine the effects of neutralising antibodies to several integrins expressed by human dermal fibroblasts *in vitro*[15]. Our data indicate that the monoclonal antibody recognising the α_2 integrin subunit inhibited cell migration induced by IGDS, but did not affect initial IGDS signalling (Table 1B). This observation is consistent with the role of $\alpha_2\beta_1$ in mediating cell attachment to collagen and its involvement in supporting cell migration on this substratum[16]. Antibodies to the $\alpha_v\beta_3$ heterodimer, as well as to the integrin subunits α_1 and β_3, were found to block both the initial events of IGDS signalling and subsequent cell migration. The $\alpha_v\beta_3$ heterodimer recognises the RGDS motif, whilst the β_1 and β_3 subunits are present in several integrin heterodimers which also recognise RGDS[15,17]. Several previous studies have implicated these integrins in the mediation of cell migration[16,18]. In this context, it should be noted that the $\alpha_v\beta_3$ heterodimer also binds to exposed RGD sites in denatured (but not native) collagen[19]; this differential ligation of denatured and native type I collagen by $\alpha_v\beta_3$ may contribute to the substratum-dependent nature of fibroblast migratory response to IGD-containing peptides reported here. Two antibodies to the "classic" fibronectin-binding $\alpha_5\beta_1$ integrin had no effect on IGDS-induced cell migration (Table 1B). These observations are consistent with previous reports suggesting that integrin $\alpha_5\beta_1$ preferentially mediates cell adhesion rather than migration[20]. Recent studies have underscored the interplay between substratum, ligand concentration and integrin function in the control of cell migration[21,22]; these complex factors

will need to be taken into account in identifying the precise integrin receptors involved in IGD ligation and the post-ligation events leading to the resultant substratum-dependent biological activity.

Results obtained with signal transduction inhibitors indicate that the tyrosine kinase inhibitor *Genistein* selectively blocked IGDS-induced cell signalling, but did not affect cell migration (Table 1C). This finding is consistent with the role of focal adhesion-associated tyrosine kinases (such as pp125FAK) in mediating integrin signal transduction[23]. The MAP kinase kinase inhibitor *PD98059* blocked both signal transduction and cell migration, in keeping with the previously reported activation of the MAP kinase cascade by integrin ligation[24]. In contrast, the PKA inhibitor blocked cell migration, but did not appear to affect initial IGDS-dependent events.

In addition to RGD, other amino acid sequences in fibronectin have been reported to mediate cell adhesion and migration; these include LDV, REDV and IDAPS[17]. All these motifs resemble RGD in that migration stimulating activity is not retained by the respective soluble synthetic peptides. The migration-stimulating activity of IGD-containing synthetic peptides appears to be unique in this sense. Although biological activity has not previously been ascribed to the conserved IGD motif in fibronectin, previous studies have implicated the ninth type I repeat (which contains the IGDS sequence) in the assembly of an extracellular fibronectin matrix[25]. The data presented here may be relevant in this context and suggest several integrins which may function in IGDS ligation.

The migration inhibiting activity of RGD-containing peptides has a number of potential clinical applications[26,27]. Structure-function studies have indicated that conservative and non-conservative amino acid substitutions,

tandem amino acid extensions and cyclicisation significantly modulate the biological activity of the RGD motif in these situations[28,29]. The converse migration-stimulating activity of IGD-containing synthetic peptides may provide an analogous platform for developing a new family of therapeutic agents which promote cell migration in clinically relevant conditions, such as impaired wound healing.

Example 2: Angiogenic response in a rat wound healing model

The IGDS peptide has been shown to stimulate fibroblast migration and elicit and angiogenic response in a rat wound healing model. In this experimental system, 1 cm^2 pieces of porcine dermal collagen films impregnated with either control medium or medium containing the indicated concentration of test substance were implanted subcutaneously into rats. The animals were sacrificed 28 days later and the removed collagen implants fixed and sectioned for image analysis. The following data were obtained, indicating that IGDS stimulated both fibroblast migration into the collagen film and an angiogenic response.

	vessels (per field)	fibroblasts (% field)
control	15.3 ± 5.5	8.3 ± 6.7
IGDS (1 μg/ml)	26.7 ± 6.4	18.2 ± 9.4
	$p < 0.01$	$p < 0.01$

Table 1. Effects of various inhibitors on early (receptor ligation and signalling) and late persistent stimulation of cell migration) aspects of IGDS activity.

A: synthetic peptides						
peptide (μmolar)	preincubation		in assay		% inhibition	inhibitory activity yes(+) no(-)
	IGDS	peptide	IGDS	peptide		
RGDS (10.0)	-	-	+	+	92.3±2.1	
	+	+	-	-	96.2±4.8	early events: +
	+	-	-	+	96.5±5.7	late events: +
RGES (10.0)	-	-	+	+	1.2±2.1	
	+	+	-	-	-1.0±3.0	early events: -
	+	-	-	+	0.3±2.8	late events: -

B: integrin antibodies

antibody (μg/ml)	preincubation		in assay		% inhibition	inhibitory activity yes(+) no(-)
	IGDS	antibody	IGDS	antibody		
α_2	-	-	+	+	92.9±6.1	
(10.0)	+	+	-	-	-5.7±2.0	early events: -
	+	-	-	+	97.4±8.9	late events: +
β_1	-	-	+	+	96.1±2.1	
(10.0)	+	+	-	-	92.8±3.9	early events: +
	+	-	-	+	99.1±5.5	late events: +
β_3	-	-	+	+	94.0±4.2	
(5.0)	+	+	-	-	98.0±1.0	early events: +
	+	-	-	+	96.7±3.6	late events: +
$\alpha_5\beta_1$	-	-	-	+	4.1±3.3	
(10.0)	+	+	+	-	3.6±2.8	early events: -
	+	-	+	+	-0.1±2.6	late events: -
$\alpha_v\beta_3$	-	-	+	+	97.9±4.7	
(10.0)	+	+	-	-	88.0±3.3	early events: +
	+	-	-	+	68.9±12.0	late events: +

C: signal transduction inhibitors

inhibitor	preincubation		in assay		% inhibition	inhibitory activity yes(+) no(-)
	IGDS	inhibitor	IGDS	inhibitor		
Genistein (10 μg/ml)	-	-	+	+	94.1±6.5	
	+	+	-	-	96.7±4.0	early events: +
	+	-	-	+	0.9±2.9	late events: -
PD98059 (2.0 μM)	-	-	+	+	99.8±3.8	
	+	+	-	-	90.5±3.1	early events: +
	+	-	-	+	39.4±5.0	late events: +
PKA inhib (5 nM)	-	-	+	+	90.0±6.4	
	+	+	-	-	6.9±5.7	early events: -
	+	-	-	+	99.5±4.2	late events: +

Table 2. Angiogenic Activities of Synthetic Peptides and Gelatin-binding Domain of Fibronectin. The angiogenic activities of the indicated test compounds were ascertained in the chick yolk sac assay, as previously described in Gush *et al* (1990) *J. Med. Engineer. Tech.* 14, 205-209. See Legend Figure 7 for further details.

Angiogenic Activity		
Compound	**Concentration (ng/pellet)**	**Positive responses (%)**
control	-	2/28 (7)
IGDS	5	3/6 (50)
	50	7/11 (64)
	250	10/13 (77)
	1000	9/14 (64)
	3000	7/9 (78)
Gelatin-binding domain	5	0/4 (0)
	50	3/10 (30)
	250	7/8 (87)
	1000	9/12 (75)
	3000	6/6 (100)
RGDS	3000	1/11 (9)
RGES	3000	1/10 (10)

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CLAIMS

1. A compound with a relative molecular mass of less than 15000 comprising the peptide Ile-Gly-Asp (IGD) or a peptide or non-peptide mimic thereof.

2. A compound according to Claim 1 comprising the peptide Ile-Gly-Asp-Ser (IGDS) or a peptide or non-peptide mimic thereof.

3. A compound according to Claim 1 comprising the peptide Ile-Gly-Asp-Gln (IGDQ) or a peptide or non-peptide mimic thereof.

4. A compound according to any one of Claims 1 to 3 wherein the said amino acids within the said peptide are in the L-configuration.

5. A compound according to any one of Claims 1 to 4 which is a peptide comprising the peptide Ile-Gly-Asp.

6. A compound according to Claim 5 comprising the peptide Ile-Gly-Asp-Ser.

7. A compound according to Claim 5 comprising the peptide Ile-Gly-Asp-Gln.

8. A compound according to any one of the preceding claims with a relative molecular mass of less than 8000.

9. A compound according to Claim 8 with a relative molecular mass of less than 5000.

10. A compound according to Claim 9 with a relative molecular mass of less than 2000.

11. The compound Ile-Gly-Asp.

12. The compound Ile-Gly-Asp-Ser.

13. The compound Ile-Gly-Asp-Gln.

14. A compound according to any one of the preceding claims for use in medicine.

15. A pharmaceutical composition comprising a compound according to any one of the preceding claims and a pharmaceutically acceptable carrier.

16. A polynucleotide encoding a peptide according to any one of Claims 5 to 7.

17. A vector comprising a polynucleotide according to Claim 16.

18. A host cell comprising a polynucleotide according to Claim 16 or 17.

19. A method of modulating cell migration the method comprising administering an effective amount of a compound according to any one of Claims 1 to 13 to the site where modulation of cell migration is required.

20. A method according to Claim 19 wherein the cell is a fibroblast.

21. A method according to Claim 19 or 20 wherein the site is in an animal body.

22. A method according to Claim 21 wherein the site is in a mammalian.

23. A method according to Claim 22 wherein the site is in a human body.

24. Use of a compound according to any one of Claims 1 to 13 for modulating cell migration.

25. Use of a compound according to any one of Claims 1 to 13 in the manufacture of a medicament for modulating cell migration in an animal body.

26. Any novel IGD-containing compound herein disclosed.

27. Any novel use of an IGD-containing compound herein disclosed.

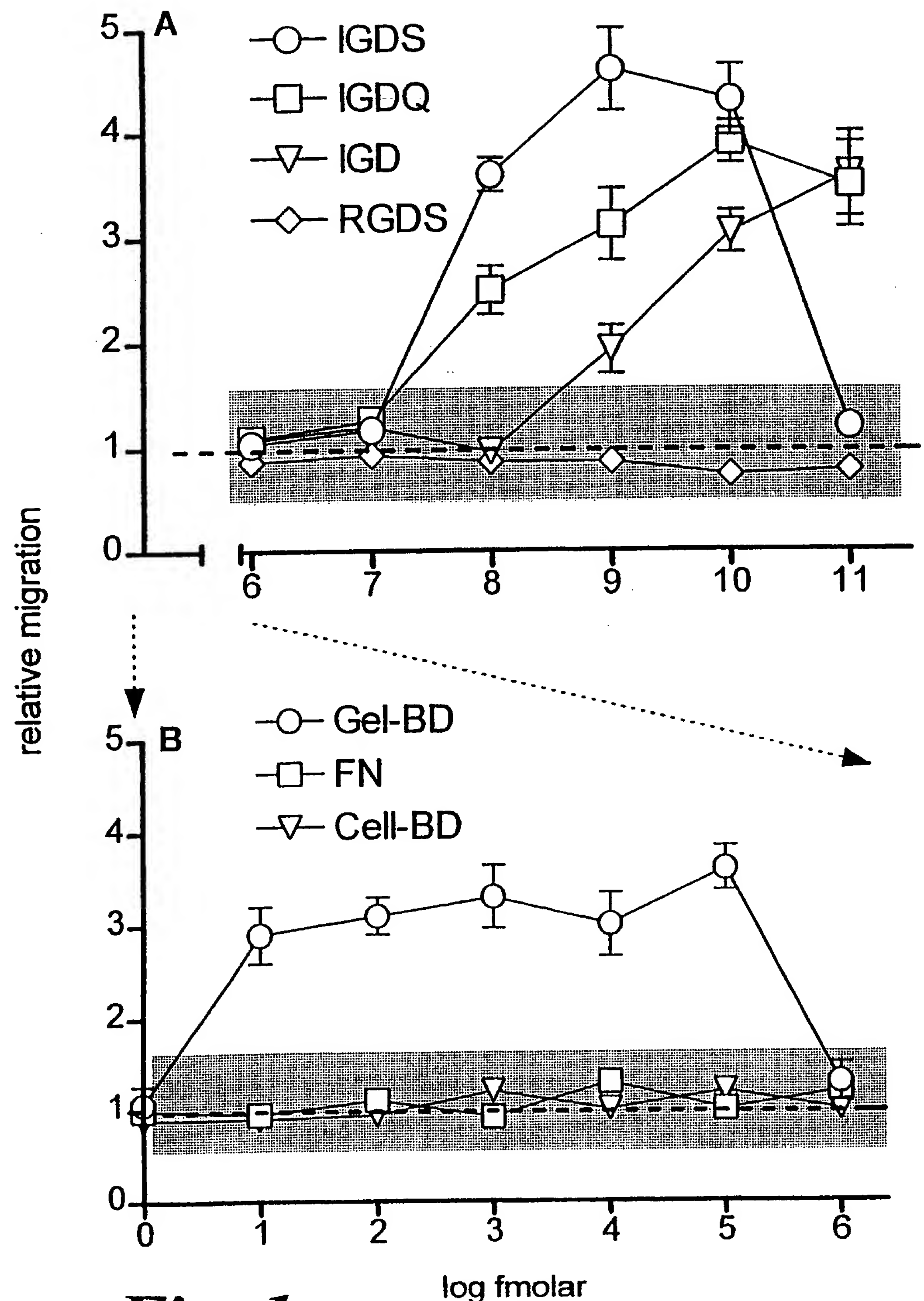
A
IGDS
IGDQ
IGD
RGDS
B
Gel-BD
FN
Cell-BD
relative migration
log fmolar
0
1
2
3
4
5
6
7
8
9
10
11

Fig. 1

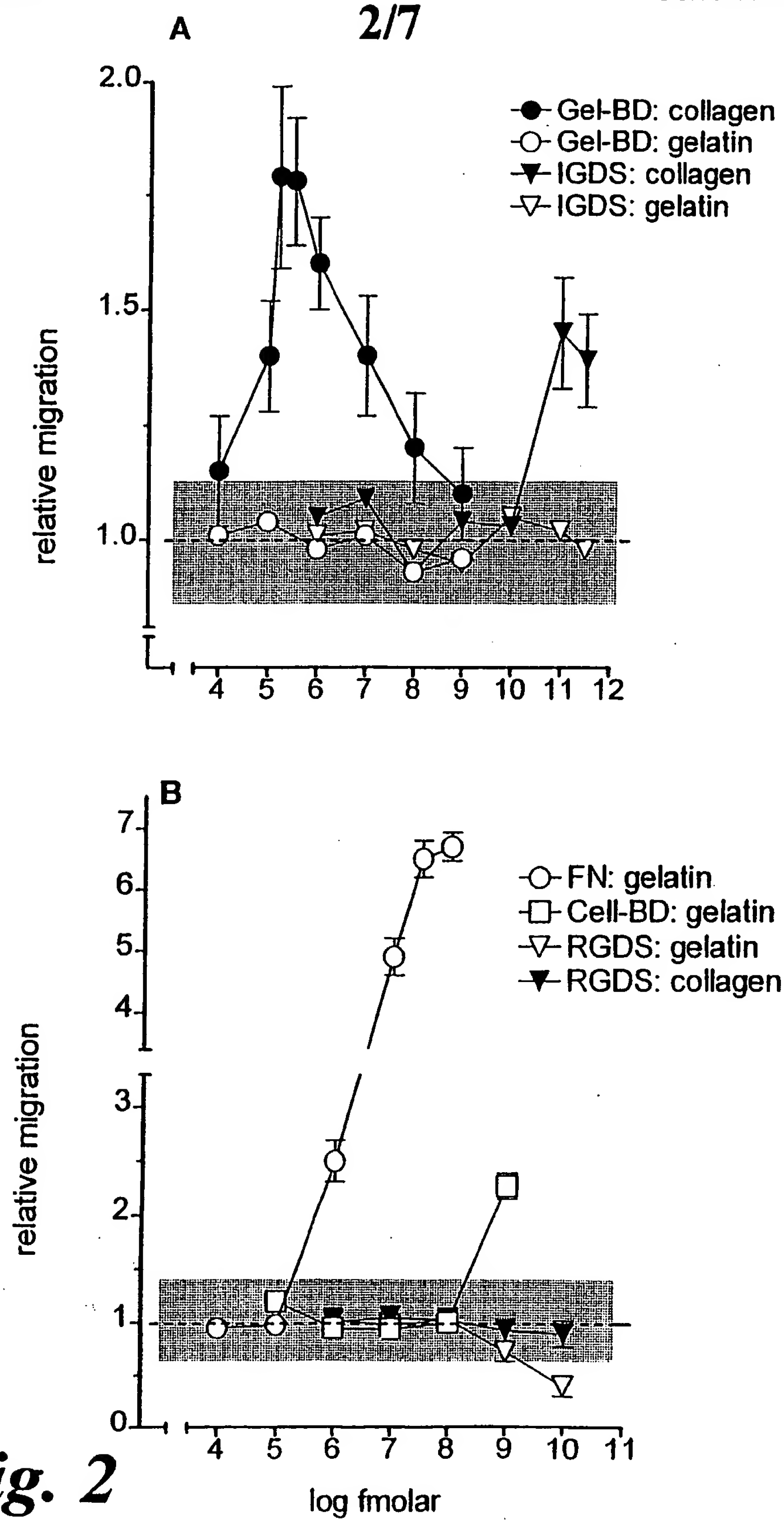
A
2.0
1.5
1.0
relative migration
Gel-BD: collagen
Gel-BD: gelatin
IGDS: collagen
IGDS: gelatin
4 5 6 7 8 9 10 11 12
B
7
6
5
4
3
2
1
0
relative migration
FN: gelatin
Cell-BD: gelatin
RGDS: gelatin
RGDS: collagen
4 5 6 7 8 9 10 11
log fmolar

Fig. 2

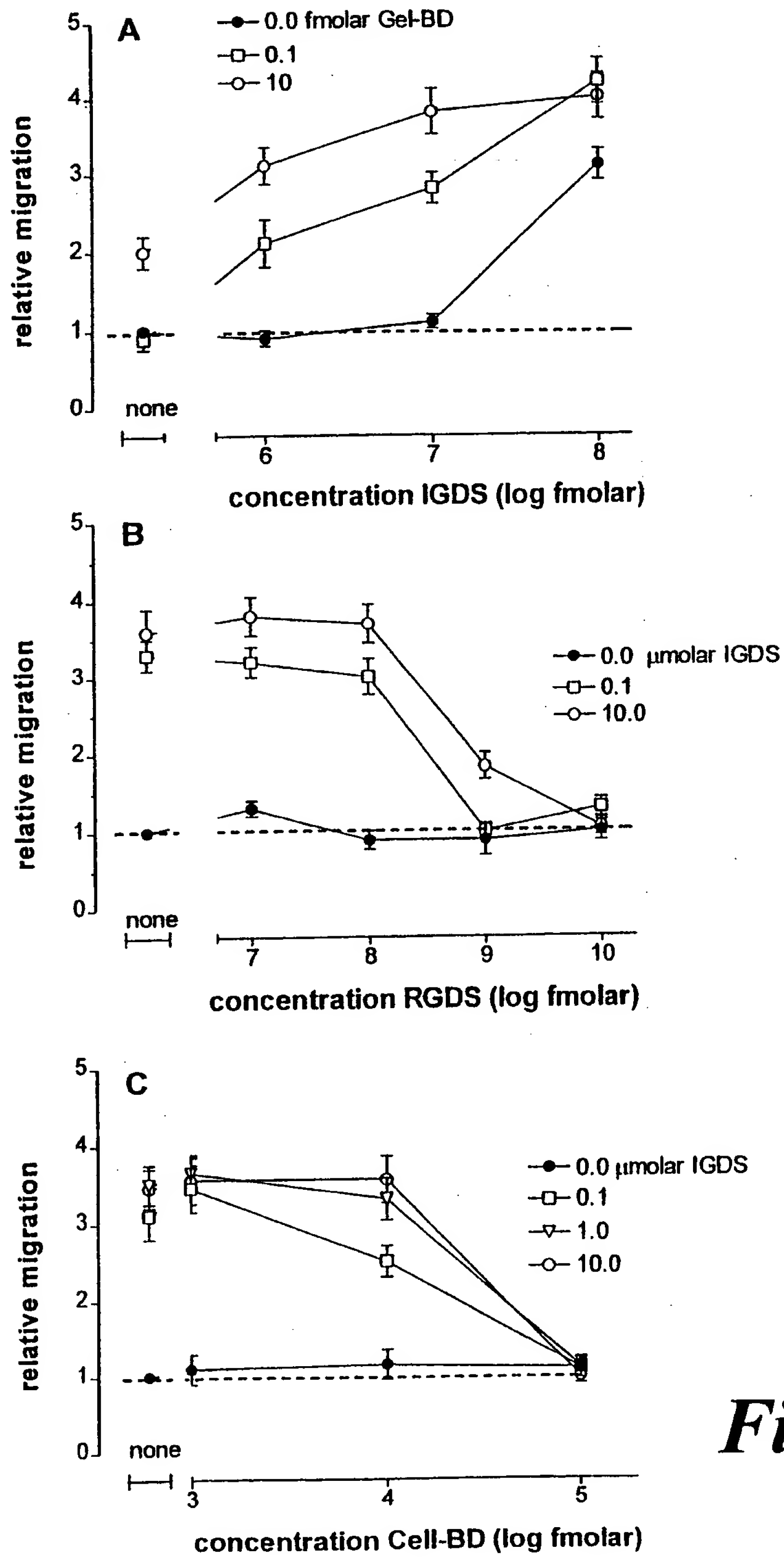
A
0.0 fmolar Gel-BD
0.1
10
relative migration
none
concentration IGDS (log fmolar)
B
0.0 μmolar IGDS
0.1
10.0
relative migration
none
concentration RGDS (log fmolar)
C
0.0 μmolar IGDS
0.1
1.0
10.0
relative migration
none
concentration Cell-BD (log fmolar)

Fig. 3

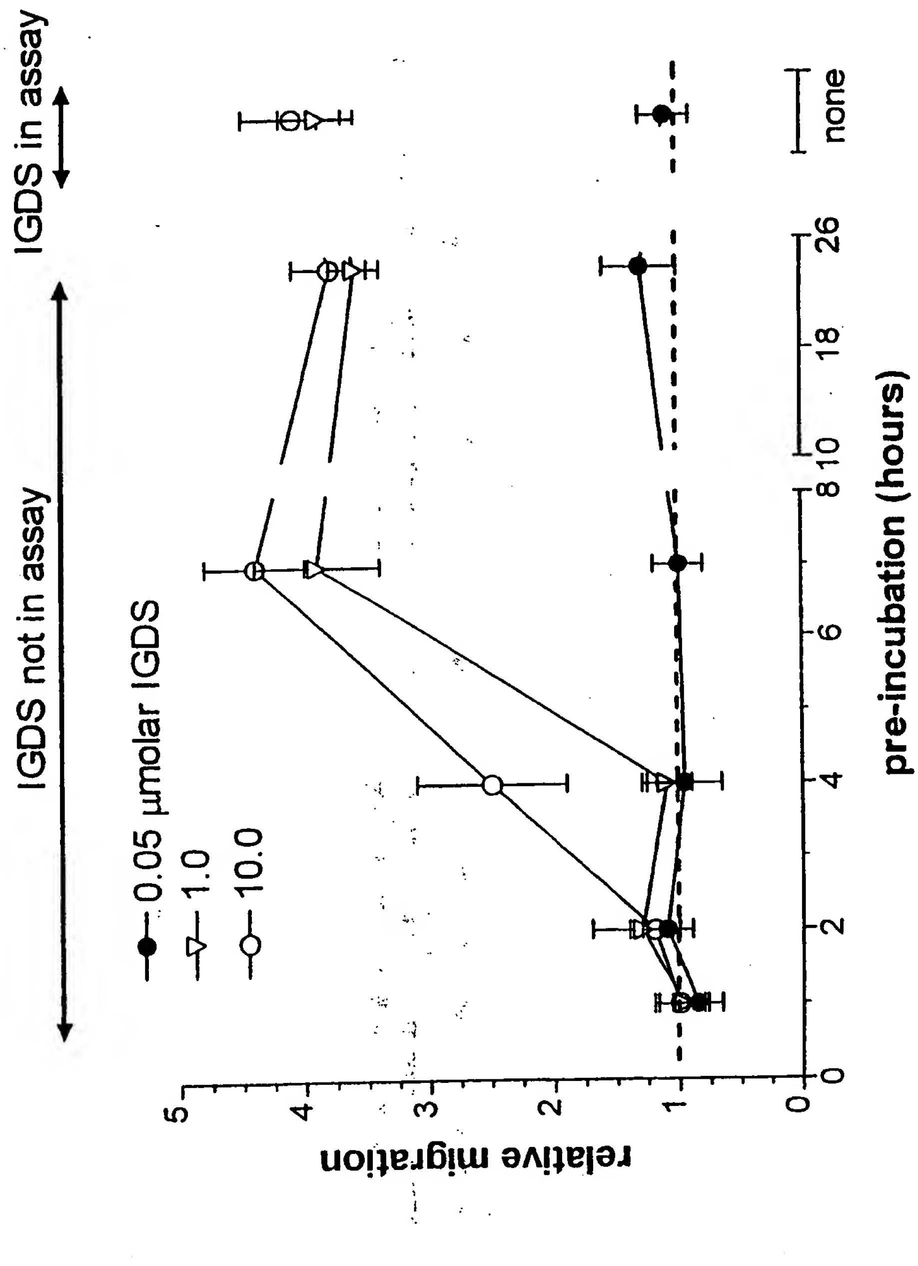
IGDS not in assay
IGDS in assay
0.05 μmolar IGDS
1.0
10.0
relative migration
0
1
2
3
4
5
0
2
4
6
8
10
18
26
none
pre-incubation (hours)

Fig. 4

1	MLRGPGPGLL	LLAVQCLGTA	VPSTGASKSK	RQAQQMVQPQ	SPVAVSQSKP
51	GCYDNGKHYQ	INQQWERTYL	GNVLVCTCYG	GSRGFNCESK	PEAEETCFDK
101	YTGNTYRVGD	TYERPKDSMI	WDCTCIGAGR	GRISCTIANR	CHEGGQSYKI
151	GDTWRRPHET	GGYMLECVCL	GNGKGEWTCK	PIAEKCFDHA	AGTSYVVGET
201	WEKPYQGWMM	VDCTCLGEGS	GRITCTSRNR	CNDQDTRTSY	RIGDTWSKKD
251	NRGNLLQCIC	TGNGRGEWKC	ERHTSVQTTS	SGSGPFTDVR	AAVYQPQPHP
301	QPPPYGHCVT	DSGVVYSVGM	QWLKTQGNKQ	MLCTCLGNGV	SCQETAVTQT
351	YGGNSNGEPC	VLPFTYNGRT	FYSCTTEGRQ	DGHLWCSTTS	NYEQDQKYSF
401	CTDHTVLVQT	QGGNSNGALC	HFPFLYNNHN	YTDCTSEGRR	DNMKWCGTTQ
451	NYDADQKFGF	CPMAAHEEIC	TTNEGVMYRI	GDQWDKQHDM	GHMMRCTCVG
501	NGRGEWTCYA	YSQLRDQCIV	DDITYNVNDT	FHKRHEEGHM	LNCTCFGQGR
551	GRWKCDPVDQ	CQDSETGTFY	QIGDSWEKYV	HGVRYQCYCY	GRGIGEWHCQ
601	PLQTYPSSSG	PVEVFITETP	SQPNSHPIQW	NAPQPSHISK	YILRWRPKNS
651	VGRWKEATIP	GHLNSYTIKG	LKPGVVYEGQ	LISIQQYGHQ	EVTRFDFTTT
701	STSTPVTSNT	VTGETTPFSP	LVATSESVTE	ITASSFVVSW	VSASDTVSGF
751	RVEYELSEEG	DEPQYLDLPS	TATSVNIPDL	LPGRKYIVNV	YQISEDGEQS
801	LILSTSQTTA	PDAPPDPTVD	QVDDTSIVVR	WSRPQAPITG	YRIVYSPSVE
851	GSSTELNLPE	TANSVTLSDL	QPGVQYNITI	YAVEENQEST	PVVIQQETTG
901	TPRSDTVPSP	RDLQFVEVTD	VKVTIMWTPP	ESAVTGYRVD	VIPVNLPGEH
951	GQRLPISRNT	FAEVTGLSPG	VTYYFKVFAV	SHGRESKPLT	AQQTTKLDAP
1001	TNLQFVNETD	STVLVRWTPP	RAQITGYRLT	VGLTRRGQPR	QYNVGPSVSK
1051	YPLRNLQPAS	EYTVSLVAIK	GNQESPKATG	VFTTLQPGSS	IPPYNTEVTE
1101	TTIVITWTPA	PRIGFKLGVR	PSQGGEAPRE	VTSDSGSIVV	SGLTPGVEYV
1151	YTIQVLRDGQ	ERDAPIVNKV	VTPLSPPTNL	HLEANPDTGV	LTVSWERSTT
1201	PDITGYRITT	TPTNGQQGNS	LEEVVHADQS	SCTFDNLSPG	LEYNVSVYTV
1251	KDDKESVPIS	DTIIPEVPQL	TDLSFVDITD	SSIGLRWTPL	NSSTIIGYRI
1301	TVVAAGEGIP	IFEDFVDSSV	GYYTVTGLEP	GIDYDISVIT	LINGGESAPT
1351	TLTQQTAVPP	PTDLRFTNIG	PDTMRVTWAP	PPSIDLTNFL	VRYSPVKNEE
1401	DVAELSISPS	DNAVVLTNLL	PGTEYVVSVS	SVYEQHESTP	LRGRQKTGLD
1451	SPTGIDFSDI	TANSFTVHWI	APRATITGYR	IRHHPEHFSG	RPREDRVPHS
1501	RNSITLTNLT	PGTEYVVSIV	ALNGREESPL	LIGQQSTVSD	VPRDLEVVAA
1551	TPTSLLISWD	APAVTVRYYR	ITYGETGGNS	PVQEFTVPGS	KSTATISGLK
1601	PGVDYTITVY	AVTGRGDSPA	SSKPISINYR	TEIDKPSQMQ	VTDVQDNSIS
1651	VKWLPSSSPV	TGYRVTTTPK	NGPGPTKTKT	AGPDQTEMTI	EGLQPTVEYV
1701	VSVYAQNPSG	ESQPLVQTAV	TNIDRPKGLA	FTDVDVDSIK	IAWESPQGQV
1751	SRYRVTYSSP	EDGIHELFPA	PDGEEDTAEL	QGLRPGSEYT	VSVVALHDDM
1801	ESQPLIGTQS	TAIPAPTDLK	FTQVTPTSLS	AQWTPPNVQL	TGYRVRVTPK
1851	EKTGPMKEIN	LAPDSSSVVV	SGLMVATKYE	VSVYALKDTL	TSRPAQGVVT
1901	TLENVSPPRR	ARVTDATETT	ITISWRTKTE	TITGFQVDAV	PANGQTPIQR
1951	TIKPDVRSYT	ITGLQPGTDY	KIYLYTLNDN	ARSSPVVIDA	STAIDAPSNL
2001	RFLATTPNSL	LVSWQPPRAR	ITGYIIKYEK	PGSPPREVVP	RPRPGVTEAT
2051	ITGLEPGTEY	TIYVIALKNN	QKSEPLIGRK	KTDELPQLVT	LPHPNLHGPE
2101	ILDVPSTVQK	TPFVTHPGYD	TGNGIQLPGT	SGQQPSVGQQ	MIFEEHGFRR
2151	TTPPTTATPI	RHRPRPYPPN	VGEEIQIGHI	PREDVDYHLY	PHGPGLNPNA
2201	STGQEALSQT	TISWAPFQDT	SEYIISCHPV	GTDEEPLQFR	VPGTSTSATL
2251	TGLTRGATYN	IIVEALKDQQ	RHKVREEVVT	VGNSVNEGLN	QPTDDSCFDP
2301	YTVSHYAVGD	EWERMSESGF	KLLCQCLGFG	SGHFRCDSSR	WCHDNGVNYK
2351	IGEKWDRQGE	NGQMMSCTCL	GNGKGEFKCD	PHEATCYDDG	KTYHVGEQWQ
2401	KEYLGAICSC	TCFGGQRGWR	CDNCRRPGGE	PSPEGTTGQS	YNQYSQRYHQ
2451	RTNTNVNCPI	ECFMPLDVQA	DREDSRE		

Fig. 5

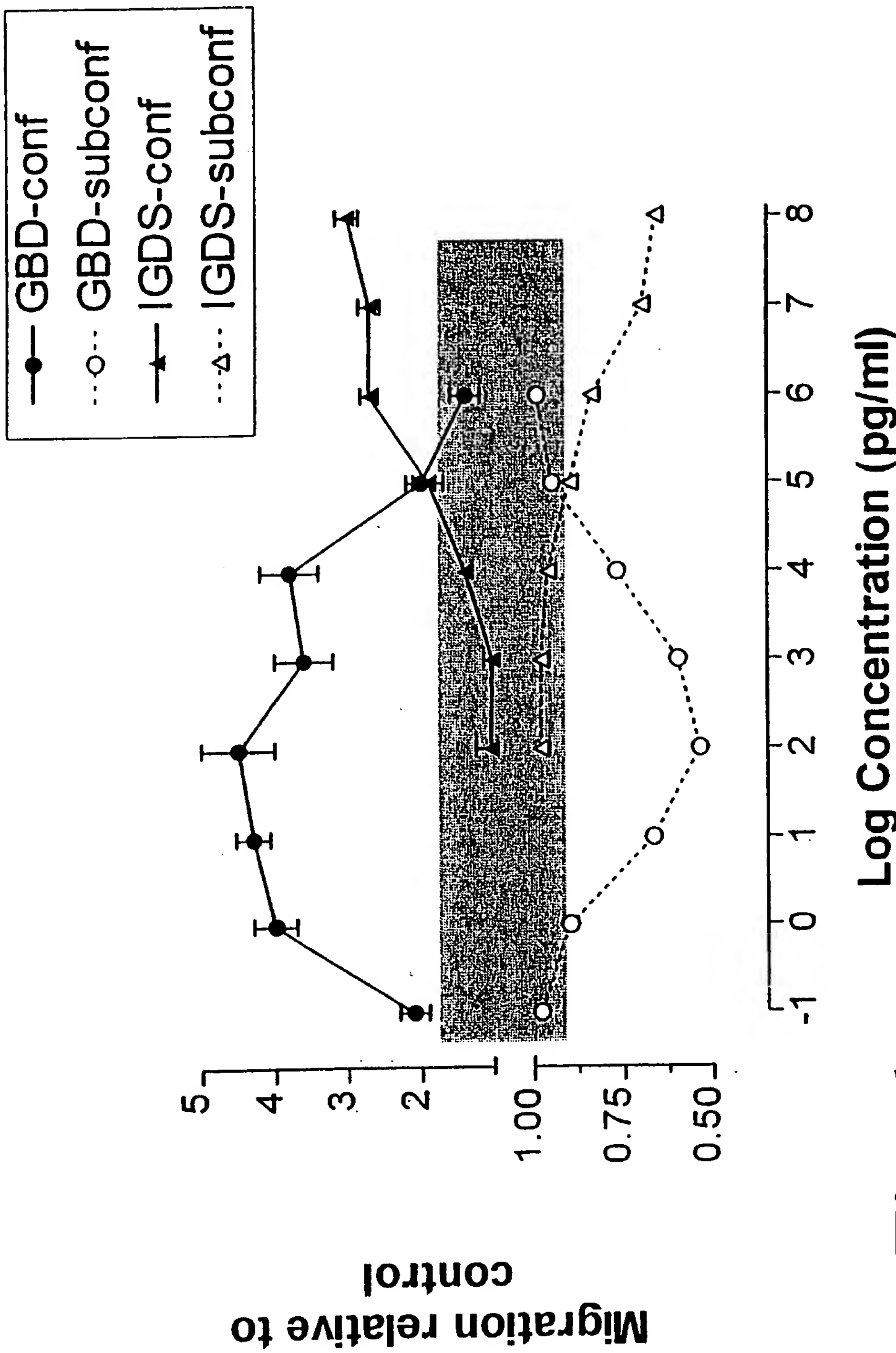
GBD-conf
GBD-subconf
IGDS-conf
IGDS-subconf
Migration relative to control
5
4
3
2
1.00
0.75
0.50
-1
0
1
2
3
4
5
6
7
8
Log Concentration (pg/ml)

Fig. 6

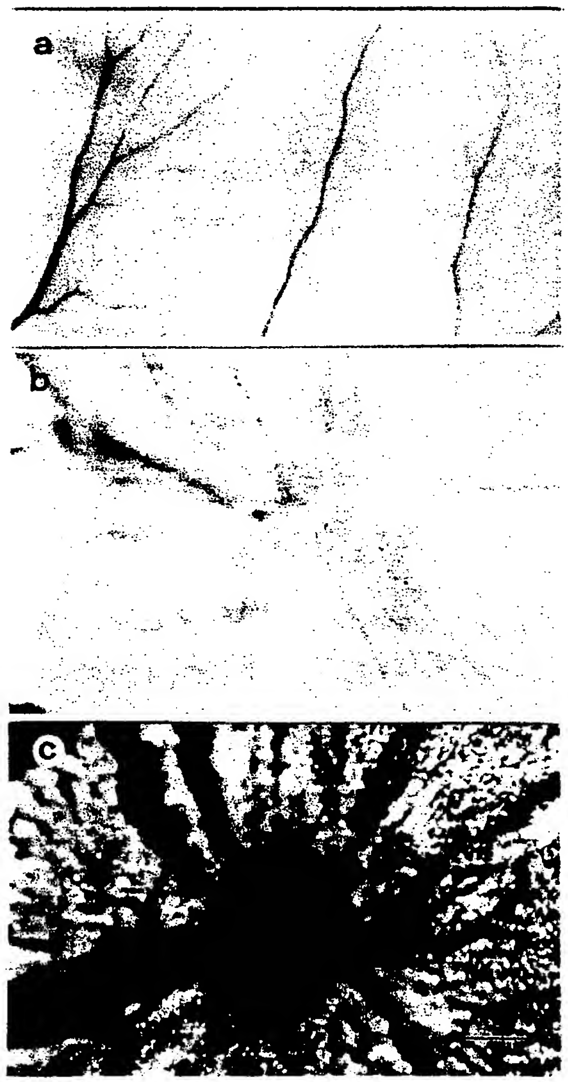

Fig. 7

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/01939

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 C07K5/08 C07K5/10 C07K14/78 A61K38/04
A61K38/39

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 39834 A (NEW YORK UNIVERSITY) 19 December 1996 see throughout, especially seq, no. 7 ---	1,2,4-6, 8,9, 14-25
X	R LOBB ET AL.: "Expression and functional characterization of a soluble form of vascular cell addhesion molecule 1" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS., vol. 178, no. 3, 15 August 1991, pages 1498-1504, XP002082124 ORLANDO, FL US see page 1500, sequence reported on last line --- -/--	1,2,4-6, 8,9, 14-25

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 October 1998	11/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Masturzo, P

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	C PANTALONI ET AL.: "Alternative splicing in the N-terminal extracellular domain of the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor modulates receptor selectivity and relative potency of PACAP-27 and PACAP-28 in phospholipase C activation " JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 271, no. 36, 6 September 1996, pages 22146-22151, XP002082125 MD US see figure 1 ---	1,2,4-6, 8,9, 14-25
X	S N SHCHELKUNOV ET AL.: "Genes of variola and vaccinia viruses necessary to overcome the host protective mechanism" FEBS LETTERS, vol. 319, no. 1,2, March 1993, pages 80-83, XP002082126 AMSTERDAM NL see the whole document ---	1-5, 14-25
X	CHEMICAL ABSTRACTS, vol. 118, no. 23, 7 June 1993 Columbus, Ohio, US; abstract no. 232073, S GRAHAM ET AL.: "Analysis of the human T-cell response to picornaviruses: identification of T-ccell epitopes close to B-cell epitopes in poliovirus" XP002082128 & J. VIROL., vol. 67, no. 3, 1993, pages 1627-637, see abstract ---	1,2,4-6, 8,9, 14-25
X	CHEMICAL ABSTRACTS, vol. 125, no. 15, 7 October 1996 Columbus, Ohio, US; abstract no. 191710, CHENG-WU CHI ET AL.: "Assignment of the binding site for tissue plasminogen activator on human fibronectin" XP002082129 & BLOOD COAGULATION, FIBRINOLYSIS, PLATELETS (JPN. CHIN. SYMP. BLOOD COAGULATION, FIBRINOLYSIS, PLATELETS), 3RD, pages 83-89, 1994 see abstract --- -/--	1,2,4-6, 8,9, 14-25

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/01939

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
P,X	CHEMICAL ABSTRACTS, vol. 128, no. 9, 2 March 1998 Columbus, Ohio, US; abstract no. 101039, R INOUE ET AL.: "Identification of Dermatophagoides farinae-s-derived peptides and class II HLA molecules recognized by T-cells from atopic individuals" XP002082130 & INT. ARCH. ALLERGY IMMUNOL., vol. 114, no. 4, April 1997, pages 354-360, see abstract	1,2,4-6, 8,9, 14-25
X	CHEMICAL ABSTRACTS, vol. 111, no. 25, 18 December 1989 Columbus, Ohio, US; abstract no. 226113, J OBERTO ET AL.: "Structure and function of the nun gene and the immunity region of the lambdoid phage HK022" XP002082131 & J MOL BIOL, vol. 207, no. 4, 1989, pages 675-693, see abstract	1,2,4-6, 8,9, 14-25
X	CHEMICAL ABSTRACTS, vol. 119, no. 11, 13 September 1993 Columbus, Ohio, US; abstract no. 110167, B AGUADO ET AL.: "Nucleotide sequence of 21.8 kbp of variola major virus strain Harvey and comparison with vaccinia virus" XP002082132 & J GEN VIROL, vol. 73, no. 11, 1992, pages 2887-2902, see abstract	1-6,8,9, 14-25
X	J WANG ET AL.: "The crystal structure of an N-terminal two-domain fragment of vascular cell adhesion molecule 1 (VCAM-1): a cyclic peptide based on the domain 1 C-D loop can inhibit VCAM-1-alpha4 integrin interaction" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 92, no. 12, 6 June 1995, pages 5714-5718, XP002082127 WASHINGTON US see the whole document	1,2,4-6, 8,9
P,X	WO 97 41731 A (ANTEX BIOLOGICS) 13 November 1997 see especially sequence no. 1	1,2

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 98/01939

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 19-24 (at least partially) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int tional Application No
PCT/GB 98/01939

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9639834 A	19-12-1996	AU 6112996 A	30-12-1996
		CA 2222690 A	19-12-1996
		EP 0843516 A	27-05-1998
WO 9741731 A	13-11-1997	AU 3118097 A	26-11-1997

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